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Abstract

Aims: The current pandemic coronavirus SARS-CoV-2 infects a wide age-group but predominantly elderly individuals, especially men and those with cardiovascular disease. Recent reports suggest an association with use of renin-angiotensin-aldosterone system (RAAS) inhibitors. Angiotensin-converting enzyme 2 (ACE2) is a functional receptor for coronaviruses. Higher ACE2 concentrations might lead to increased vulnerability to SARS-CoV-2 in patients on RAAS-inhibitors.

Methods: We measured ACE2 concentrations in 1485 men and 537 women with heart failure (Index cohort). Results were validated in 1123 men and 575 women (Validation cohort).

Results: The median age was 69 years for men, and 75 years for women. The strongest predictor of elevated concentrations of ACE2 in both cohorts was male sex (estimate=0.26, $p<0.001$ and 0.19; $p<0.001$ respectively). In the index cohort, use of ACE-inhibitors, angiotensin receptor blockers (ARBs) or mineralocorticoid receptor antagonists (MRAs) were not independent predictors of plasma ACE2. In the validation cohort, ACE-inhibitor (estimate=-0.17; p=0.002) and ARB use (estimate=-0.15; p=0.03) were independent predictors of lower plasma ACE2, while use of a MRA (estimate=0.11; p=0.04) was an independent predictor of higher plasma ACE2 concentrations.

Conclusion: In two independent cohorts of patients with heart failure, plasma concentrations of ACE2 were higher in men than women, but neither use of an ACE-inhibitor nor an ARB was associated with higher plasma ACE2 concentrations. These data might explain the higher incidence and fatality rate of COVID-19 in men, but do not support previous reports suggesting that ACE-inhibitors or ARBs increase the vulnerability for COVID-19 through increased plasma ACE2 concentrations.

Keywords: Men, Heart Failure, Coronavirus disease(COVID-19), ACE2

Introduction

The world is currently faced with the outbreak of a new Severe Acute Respiratory Syndrome coronavirus (SARS-CoV). The new virus, SARS-CoV-2 emerged in December 2019 in the city of Wuhan China, and is the causative agent of a respiratory syndrome now known as coronavirus disease 2019 (COVID-19)[1, 2]. Efforts aimed at curbing the spread of SARS-CoV-2 and finding effective treatments are ongoing.

Early epidemiological observations indicate that SARS-CoV-2 infects all age-groups, but older men with chronic illnesses may be more severely affected. There is a preponderance of men (58.1%) compared to women (41.9%) testing positive for COVID-19[2] and in the previous SARS-CoV epidemic in 2003, men had a higher mortality than women (21.9% versus 13.2%; $p<0.0001$)[3]. Whether men with the current SARS-CoV-2 virus also have a worse mortality outcome is becoming apparent as recent report indicate that 70% of patients that died of COVID-19 in Italy were men[4]; and mainly elderly.

The increased vulnerability of older people with cardiovascular disease and comorbid conditions could be related to increased concentrations of angiotensin-converting enzyme 2 (ACE2)[5, 6], and ACE2 is known to be increased in heart failure[7]. ACE2 is not only an enzyme but also a functional receptor on cell-surfaces for both SARS-CoV and SARS-CoV-2 and is highly expressed in the heart, testis, kidneys and lungs[8-12] and shed into the plasma. Some reports have suggested that inhibitors of the renin-angiotensin-aldosterone system (RAAS) increase plasma ACE2 concentrations[5, 13], although these speculations are not supported by a substantial body of research.

We therefore investigated plasma concentrations of ACE2 in two large and independent cohorts of men and women with heart failure according to the use of renin-angiotensin-aldosterone-inhibitors.

Methods

Study participants

From the BIOlogy Study to TAilored Treatment in Chronic Heart Failure (BIOSTAT-CHF) [14], we measured ACE2 concentrations in 1485 men and 537 women with heart failure in 11 European countries. Results were validated in another, independent BIOSTAT-CHF cohort consisting of 1123 men and 575 women with heart failure enrolled in Scotland. Only participants with sufficient plasma samples were used for this research. The design and baseline characteristics of both cohorts of BIOSTAT-CHF have been published elsewhere[14]. Inclusion criteria were the same in the index and validation cohorts; the only exception was that when the left ventricular ejection fraction (LVEF) was > 40%, patients had to have a BNP >400ng/L or NT-proBNP >2,000ng/L in the index cohort, but not in the validation cohort. The study complied with the Declaration of Helsinki and was approved by the medical ethics committees of participating centers[14].

ACE2 was measured using the Olink Proseek analysis service (Olink Proteomics, Uppsala, Sweden). The Olink platform[15] utilizes a high-throughput multiplex immunoassay based on a proprietary Proximity Extension Assay (PEA) technology, where each biomarker is addressed by a matched pair of antibodies, coupled to unique, partially complementary oligonucleotides, and measured by quantitative real-time polymerase chain reaction (PCR). Results are expressed in the form of relative quantification (Normalized Protein eXpression or NPX) which are logarithmically related to protein concentration but cannot be converted to absolute protein concentrations. Interpretations are therefore relative and not absolute. Analytical validation of the sensitivity and specificity of the Olink assay for this study was achieved by comparing available routine laboratory measurements of two protein biomarkers, GDF15

(pg/mL) and NT-proBNP (pg/mL) with those measured using Olink (NPX). NT-proBNP is a canonical biomarker in cardiovascular disease biology[16].

Statistical Analyses

All statistical analyses were performed using R[17] version 3.6.2. In group comparisons, categorical variables were depicted as numbers with percentages. Normally distributed variables were depicted as means ± standard deviation, non-normally distributed variables as median and interquartile range (IQR) defined as the first and third quartile (Q1–Q3). The means for continuous variables were compared by one-way analysis of variance (ANOVA) or the Kruskal–Wallis test, while categorical variables were compared by the Chi-squared test. Multivariate models were based on backward stepwise regression. Baseline tables were made using the R-based CompareGroups[18] package. In general, a two-tailed p-value of <0.05 was considered statistically significant.

Results

Clinical characteristics

Baseline characteristics of the index and validation cohort are presented in table 1 and supplemental table 1 respectively. In the index cohort (n=2022), the median age was 69 years in men (interquartile range, IQR, 60-76), and for women 75 years (IQR 64-81; p<0001 between men and women). In the validation cohort (n=1698), the median age for men was 74 years (IQR 66-81) and for women 76 years (IQR 69-82; p< 0.001 between men and women). In the index cohort, patients with higher concentrations of ACE2 were more often men, were more likely to have atrial fibrillation and a higher heart rate and lower systolic blood pressure (table 1). In the validation cohort, patients with higher concentrations of ACE2 were more

often men, were more likely to have atrial fibrillation and diabetes and a higher heart rate and lower systolic blood pressure (supplemental table 1). In the index cohort, only 0.3% (6/2022) patients received both ACEi and ARB. In the validation cohort only 0.4% (7/1691) received both ACEi and ARB.

Among patients that were not treated with RAAS-inhibitors, men were predominant in the uppermost quartile of ACE2 (supplemental table 2 and 3). ACE2 concentrations were higher in men than women in 9/11 countries but were similar by ACEi/ARB use (Supplemental Figure 1 and 2).

Analytical validation of the Olink assay

In both study cohorts, routine lab concentrations of two golden standard biomarkers (GDF-15 and NT-proBNP) showed a strong correlation with those measured using Olink (Spearman's rho 0.77-0.92, $p<0.001$; Supplemental Figure 3).

ACE2 concentrations in men and women according to the use of RAAS-inhibitors

The ACE2-RAAS-COVID-19 axis is summarized in Figure 1. In both cohorts, plasma ACE2 concentrations (in NPX units) were higher in men than in women. In the index cohort, mean plasma concentration was 5.38 in men compared with 5.09 in women ($p<0.001$). In the validation cohort, mean plasma concentration was 5.46 in men compared with 5.16 in women ($p<0.001$).

Figure 2 shows plasma ACE2 concentrations in those treated with or without blockers of the renin-angiotensin-aldosterone system. In the index cohort, mean plasma concentration was 5.32 in patients who used an ACE-inhibitor compared with 5.29 in those who did not ($p = 0.59$. In the validation cohort, mean plasma concentration was 5.32 in those who used an ACE-inhibitor versus 5.4 in those who did not ($p = 0.0033$). In the index cohort, mean plasma

concentration was 5.23 in patients who used an ARB compared with 5.31 in those who did not (p = 0.16). In the validation cohort, mean plasma concentration was 5.3 in those who used an ARB versus 5.37 in those who did not (p = 0.38). In the index cohort, mean plasma concentration was 5.35 in patients who used an MRA compared with 5.25 in those who did not (p = 0.003). In the validation cohort, mean plasma concentration was 5.4 in those who used an MRA versus 5.34 in those who did not (p = 0.036).

Age and sex interaction analyses indicated that men who used MRA have an increased ACE2 concentration ($p \leq 0.01$, for models unadjusted, and those adjusted for ACEi use, ARB use, age, diabetes and atrial fibrillation). This was statistically significant only in the index cohort. In the validation cohort, men who used ACEi had lower ACE2 concentrations ($p<0.05$; for models unadjusted, and those adjusted for ACEi use, ARB use, age, diabetes and atrial fibrillation). All similar interaction tests were not statistically significant.

Variables associated with plasma ACE2 concentrations

The strongest predictor of elevated plasma concentrations of ACE2 in the index and validation cohort was male sex (estimate=0.26, $p<0.001$ and 0.19; $p<0.001$ respectively). In the index cohort, neither ACE-inhibitors, ARBs nor MRAs were associated with plasma ACE2 concentrations (table 2). In the validation cohort, ACE-inhibitors (estimate=-0.17; p=0.002) and ARBs (estimate=-0.15;p=0.03) were associated with lower plasma ACE2 concentrations, but MRAs (estimate=0.11;p=0.04) were associated with higher concentrations (supplemental table 4).

Discussion

In two large independent cohorts of patients with heart failure, we found that plasma ACE2 concentrations were higher in men than in women. In addition, those receiving ACE-inhibitors or ARBs did not have higher concentrations of ACE2 and an increase in those taking MRA in the validation cohort was not confirmed in the index cohort.

There is an increased susceptibility of elderly people with chronic comorbidities to SARS coronaviruses and men appear to be especially vulnerable to SARS-CoV-2[1, 2, 19]. Given that a typical heart failure patient belongs to this high-risk group, we sought to uncover factors that could explain the sex-based susceptibility to SARS-CoV-2 in this vulnerable population.

Baseline characteristics of the two cohorts presented are typical for patients with heart failure and confirm that these are elderly patients that often have comorbidities, including diabetes, hypertension, renal disease and COPD. The spectrum of comorbidities involves most of the organs affected in COVID-19, including the heart, lungs, kidneys and liver[1].

COVID-19 patients and other patients with such underlying diseases are in a hyper-inflammatory state. As such it might well be that patients with various kidney diseases have high endothelial ACE2[20]; making ACE2 a damage marker. Furthermore, plasma ACE2 activity is increased in patients with heart failure[21].

Post-transcriptional events of ACE2 in the testis

ACE2 is widely distributed in tissues including lung alveolar epithelial cells, vascular endothelium, heart, kidney and testis[8, 11, 20, 22]. For the readers' convenience, we provide Supplemental Figures 4 and 5 showing the gene structure of ACE2, and its isoforms and tissue distribution). ACE2 protein and interestingly also the non-coding isoforms are highly expressed in the testis[22] (Supplemental Figure 6). Isoform transcription could possibly affect protein translation in this male-specific tissue; for example via MicroRNA (miRNA)

competition. Previous studies indicate that ACE2 may be subject to post-transcriptional regulation via miR-421[23] which could be exploited as a novel potential therapeutic target to modulate ACE2 expression in disease. How the testis-expressed ACE2 protein, or those expressed at other organs, enters circulation is largely unknown. The tissue-specific transcriptional regulation of ACE2 could partially explain higher ACE2 protein concentrations and why a coronavirus would flourish in men.

ACE2 plasma concentrations and use of RAAS-inhibitors

Recently, it was suggested that the higher prevalence and fatality rate in patients with cardiac diseases, such as hypertension or diabetes, was related to the concomitant use of ACE-inhibitors and ARBs that were suggested to increase ACE2 concentrations. The authors speculated that this might be due to increased expression of ACE2 but offered no evidence for this[5, 6, 24, 25]. In animal models, selective blockade of either Angiotensin II synthesis or activity induced increases in cardiac ACE2 gene expression and cardiac ACE2 activity[13], whether this translates to humans needs to be validated.

To the best of our knowledge, this is the first substantial study to examine the association between plasma ACE2 concentrations and the use of RAAS-blockers in patients with cardiovascular disease. In contrast to previous reports[5, 6, 24, 25], ACE-inhibitors and ARBs were not associated with increased plasma concentrations of ACE2 in the present study. Indeed, if anything, the use of ACE-inhibitors and ARB predicted *lowe*r concentrations of ACE2 in the validation cohort, although these findings were not replicated in the index cohort. Taken together, these data do not support withholding ACE-inhibitors or ARBs in patients at risk for SARS-CoV-2 infection.

In the validation cohort, MRA use was associated with a weak but statistically significant increase in plasma ACE2 concentrations. Univariate and multivariate-adjusted analyses indicated a significant sex-based interaction; with men on MRA having higher ACE2 concentrations. A similar association was, not found in the index cohort. The effect of MRA on plasma ACE2 is therefore not clear. One study found a trend (p=0.07) towards increased plasma ACE2 activity in patients treated with an MRA[21]. In addition, one mechanistic study using macrophages reported an increase of ACE2 activity after MRA[26] but further data are not available. Clearly, our findings do not suggest that MRAs should be discontinued in patients with heart failure, in whom coronavirus SARS-CoV-2 infection is found. Moreover, even if MRAs are consistently found to increase plasma ACE2 concentrations, it still needs to be established whether their use is associated with higher vulnerability to or more severe consequences of SARS-CoV-2 infection. MRAs are a very effective treatment for heart failure and these hypothetical effects on viral infection should be weighed carefully against their proven benefits.

The equilibrium between soluble and membrane-bound ACE2 might influence COVID-19 pathogenesis and treatment options. Previous studies indicate ADAM17 mediated ACE2 shedding[27, 28] , but how this would affect coronavirus infectivity during concomitant use of RAAS inhibitors warrants a separate research. A study on dogs with heart disease indicated that ACE2 shedding is not an important factor in the total extent of tissue-bound ACE2 activity, but rather a loss of tissue ACE2 into the circulation would tend to decrease the overall compensatory potential of ACE2[29]. Further work is required to show whether this translates to humans.

Conclusion:

In two large cohorts of patients with heart failure, plasma ACE2 concentrations were higher in men than in women, possibly reflecting higher tissue expression of this receptor for SARS coronavirus infections. This could explain why men might be more susceptible to infection with, or the consequences of, SARS-CoV-2. Patients receiving ACE-inhibitors or ARBs did not have higher plasma concentrations of ACE2 and any effect of MRA was small and inconsistent, supporting the continued use of these agents in patients with heart failure during the current SARS-CoV-2 pandemic.

Limitations

The conclusions drawn in this analysis are mainly restricted to heart failure, albeit a group of patients at high risk for COVID-19. Secondly, since our patients are not coronavirus-infected, we cannot provide a direct link between the course of COVID-19 disease in patients with low versus high plasma ACE2 concentrations, and the influence of age and RAAS-blockers on the course of the disease. Thirdly, we measured plasma ACE2 concentrations and not membrane-bound ACE2. We can only speculate that circulating concentrations are associated with tissue concentration, since there is no compelling evidence for this.

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Disclosures

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Figure Legend

Figure 1: Summary of the RAAS pathway

Figure 2: ACE2 concentrations in patients with and without use of ACEi, ARB and MRA (index and validation).

ACEi = Use of Angiotensin Converting Enzyme (ACE) inhibitors; ARB = use of Angiotensin Receptor Blocker; MRA = use of Mineralocorticoid Receptor Antagonist.

Tables

Table 1: Baseline characteristics according to quartiles of plasma ACE2 concentrations (index cohort)

Table 2: Multivariable predictors of plasma ACE2 concentrations (index cohort)

Supplemental Figure 1: Global perspective of BIOSTAT-CHF countries and mean ACE2 levels

Supplemental Figure 2: Global impression of ACE2 levels in men and women, and upon use of ACEi/ARB per country studied

Supplemental Figure 3: Analytical validation of Olink assay using routine lab measurements of GDF-15 and NT-proBNP

Supplemental Figure 4: The ACE2 gene model and exons encoding domains. (Coronavirus S1 proteins binds to the N-terminal peptidase domain of ACE2 (Source: the Ensembl genome browser))

Supplemental Figure 5: Isoforms and tissue distribution of ACE2. (Data Source: GTEx Analysis Release V8 (dbGaP Accession phs000424.v8.p2)

Supplemental Figure 6: Co-expression of ADAM17 and ACE2 at the testis and other tissues (left=RNA level; right=protein level. (Source Human Protein Atlas))

Supplemental Table 1: Baseline characteristics according to quartiles of plasma ACE2 concentrations (validation cohort)

Supplemental Table 2: Baseline characteristics according to quartiles of plasma ACE2 concentrations for patients not on RAAS-inhibitors (index cohort)

Supplemental Table 3: Baseline characteristics according to quartiles of plasma ACE2 concentrations for patients not on RAAS-inhibitors (validation cohort)

Supplemental Table 4: Multivariable predictors of plasma ACE2 concentrations (validation cohort)